Characterization of the Ligand-Binding Domain of the Ecdysteroid Receptor from Drosophila Melanogaster

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Abstract:

Mutants created by site-directed mutagenesis were used to elucidate the function of amino acids involved in ligand binding to ecdysteroid receptor (EcR) and heterodimer formation with ultraspiracle (USP). The results demonstrate the importance of the C-terminal part of the D-domain and helix 12 of EcR for hormone binding. Some amino acids are involved either in ligand binding to EcR (E476, M504, D572, I617, N626) or ligand-dependent heterodimerization as determined by gel mobility shift assays (A612, L615, T619), while others are involved in both functions (K497, E648). Some amino acids are suboptimal for ligand binding (L615, T619), but mediate ligand-dependent dimerization. We conclude that the enhanced regulatory potential by ligand-dependent modulation of dimerization in the wild type is achieved at the expense of optimal ligand binding. Mutation of amino acids (K497, E648) involved in the salt bridge between helix 4 and 12 impair ligand binding to EcR more severely than hormone binding to the heterodimer, indicating that to some extent heterodimerization compensates for the deleterious effect of certain mutations. Different effects of the same point mutations on ligand binding to EcR and EcR/USP (R511, A612, L615, I617, T619, N626) indicate that the ligand-binding pocket is modified by heterodimerization.

Key words: Dimerization /Ecdysone/ Hormone/ Insect/ Nuclear receptor/ Ultraspiracle.

Article:

Introduction

Besides molting and metamorphosis, ecdysteroids regulate many aspects of development, differentiation, reproduction and behavior during the life cycle of arthropods. To date more than 100 genes have been reported to be regulated by ecdysteroids and depend upon the tissue, the developmental stage and the gene (Thummel, 1995; Spindler-Barth and Spindler, 2000; Spindler et al., 2001). Elucidation of the molecular mechanism of ecdysteroid action is therefore a central part in understanding the hormonal control of arthropod development (Koelle et al., 1991; Lezzi et al., 1999).

The ligand-binding domain (LBD) of nuclear receptors exhibits multiple functions and mediates the hormonal control of dimerization and transactivation. Hormone binding to EcR is modulated by the dimerization partner (Vögtli et al., 1999; Grebe et al., 2002) and receptor activity is further influenced by the cellular context (Elke et al., 1999; Lan et al., 1999).

A number of ecdysteroids were tested with different bioassays and ligand binding studies (Cherbas et al., 1980; Dinan, 1989), which allowed a detailed mapping of the structurally essential parts of the ligand, by CoMFA (Dinan et al., 1989). Species-specific differences in the amino acid sequence of the ecdysteroid receptor (Mouillet et al., 1997; Suhr et al., 1998), characterization of truncated receptors (Perera et al., 1999; Lezzi et al., 2002), and the use of chimeric proteins (Henrich et al., 2000) gave some hints on the functional importance of selected amino acids or receptor domains.

In contrast to vertebrate steroid hormone receptors, no crystallographic data of the ligand-binding pocket of the

ecdysteroid receptor are available, and the sequence identity of EcR to vertebrate nuclear receptors is only modest (28% to TRß and 25% to RAR; Koelle et al., 1991). Based on a generalized model of nuclear receptors (Wurtz et al., 1996, 2000), Wurtz et al. (2000) used VDR and RAR as templates to propose a three-dimensional structure of the ligand-binding domain of EcR. From the proposed model, the amino acids lining the ligand-binding pocket and presumably involved in ligand binding were predicted.

Ligand binding and dimerization mutually influence each other. From vertebrate nuclear receptors, it is known that receptor functions are modified allosterically by intra- and intermolecular interactions (Antoniewski et al., 1996; Doesburg et al., 1997; Scheller et al., 1998; Kumar et al., 1999). In this study we characterized hormone binding and dimerization of the ligand-binding domain of the Drosophila EcR and in recombinant, mutated receptors in the absence and presence of the most important dimerization partner, ultraspiracle.

Results

To identify amino acids involved in dimerization and hormone binding, the ligand-binding domain, including part of the hinge region of EcR and USP, coupled to Gal4-AD and Gal4-DBD (Figures 1 and 2) was characterized. Various truncated forms and point mutations of EcR were tested with gel mobility shift assays and hormone binding experiments.

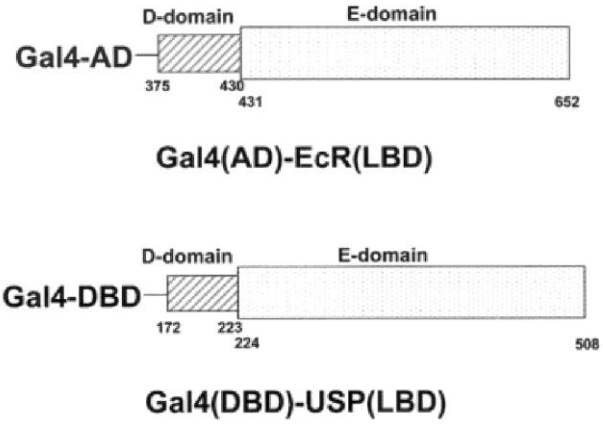

Fig. 1 Schematic Presentation of *Drosophila* EcR(LBD) and USP(LBD) Proteins Fused to GAL4 Domains Expressed in *Saccharomyces cerevisiae*.

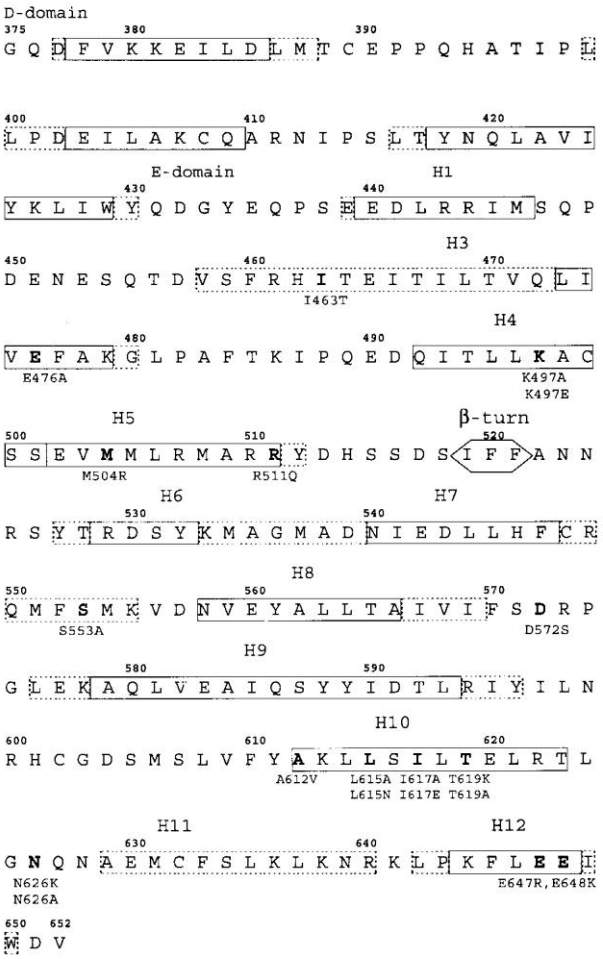

Fig. 2 Sequence of the Ligand-Binding Pocket of EcR from *Drosophila melanogaster*.
Point mutations are indicated in bold type. Helices were determined according to Spindler *et al.* (2001). Putative helices calculated by three different models (predator, predict protein using nuclear receptors with a weighted similarity of >40%, predict protein including all EcRs) are boxed. Helical structures found by only one or two methods were indicated with dashed boxes. The ß-sheets are marked with '<>'.

Characterization of the Wild-Type Ligand-Binding Domains

Heterodimers of full-length EcR and USP interact with responsive elements already in the absence of hormone, but binding is enhanced after addition of hormone (Figure 3A) as described previously (Antoniewski et al., 1996; Vögtli et al, 1998; Elke et al., 1999). With Gal4(DBD)- USP(172–508), the expected protein-DNA complex is seen using a Gal4 specific UAS (Figure 3A). No heterodimer is formed in the presence of GAl4(AD)- EcR(375–652) if hormone is omitted. In the presence of 10^{-5} M muristerone A, a shifted band is visible indicating that only ligand dependent heterodimerization is detected under these conditions. The molar ratio of USP/EcR is critical for dimerization as shown in Figure 3B.

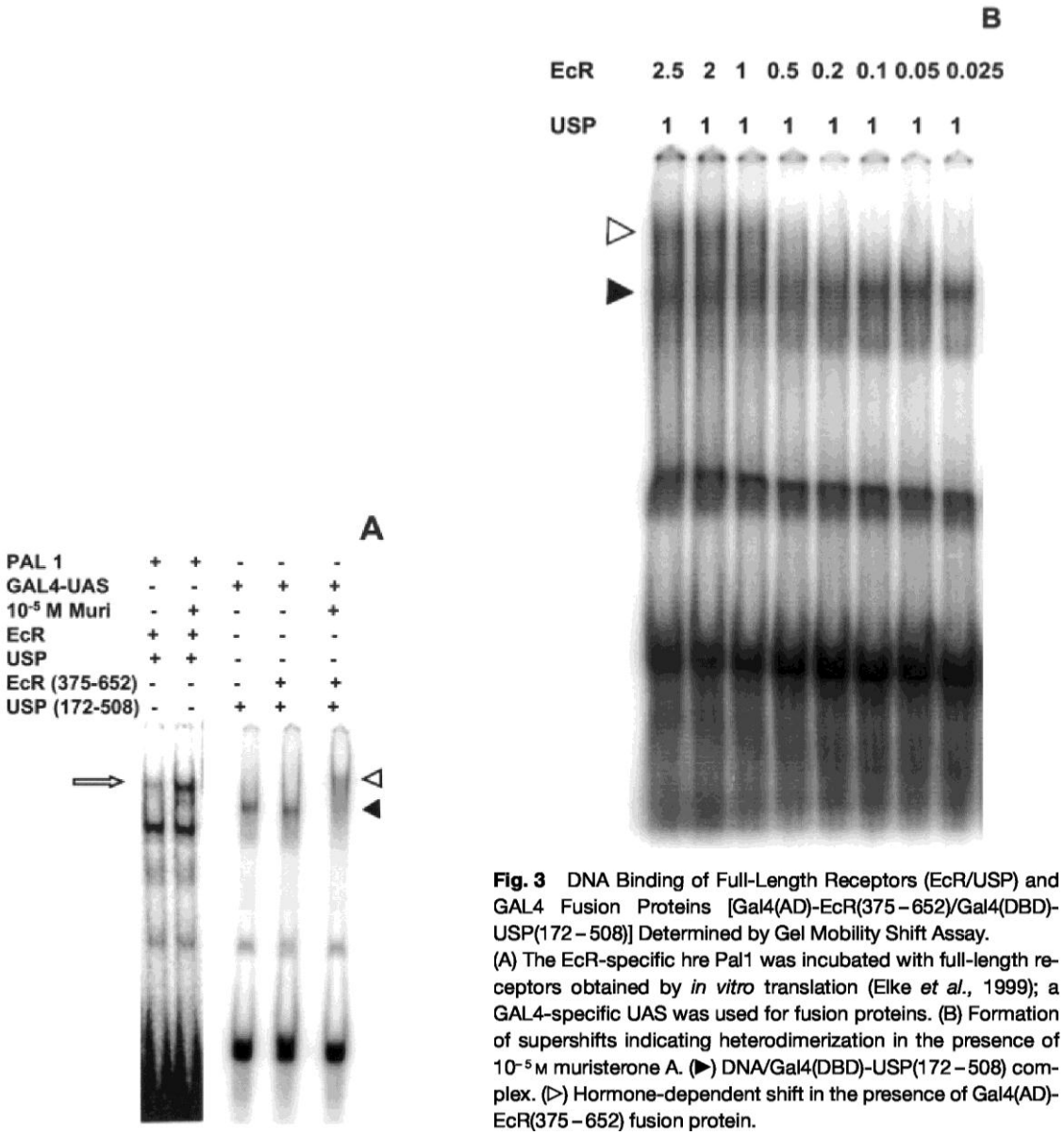

Fig. 3 DNA Binding of Full-Length Receptors (EcR/USP) and GAL4 Fusion Proteins [Gal4(AD)-EcR(375–652)/Gal4(DBD)-USP(172–508)] Determined by Gel Mobility Shift Assay.
(A) The EcR-specific hre Pal1 was incubated with full-length receptors obtained by *in vitro* translation (Elke *et al.*, 1999); a GAL4-specific UAS was used for fusion proteins. (B) Formation of supershifts indicating heterodimerization in the presence of 10^{-5} M muristerone A. (▶) DNA/Gal4(DBD)-USP(172–508) complex. (▷) Hormone-dependent shift in the presence of Gal4(AD)-EcR(375–652) fusion protein.

Wild-type, full-length receptors of Drosophila, obtained by in vitro transcription/translation (Vögtli et al., 1999), and fusion proteins containing only the C-terminal part of the hinge region and the LBD of EcR, interact with ponasterone A in the same way (Figure 4A, B). In both cases hormone already binds to a small, but significant degree in the absence of USP. In the presence of USP, ligand binding to EcR increases as much as 10-fold (Figure 4C).

To ensure optimal receptor concentrations, EcR- and USP-fusion proteins were expressed separately and their concentrations in yeast extracts were checked by quantitative evaluation of Western blots (Figure 5A), using a standard curve with wild-type receptor domains (Figure 5B).

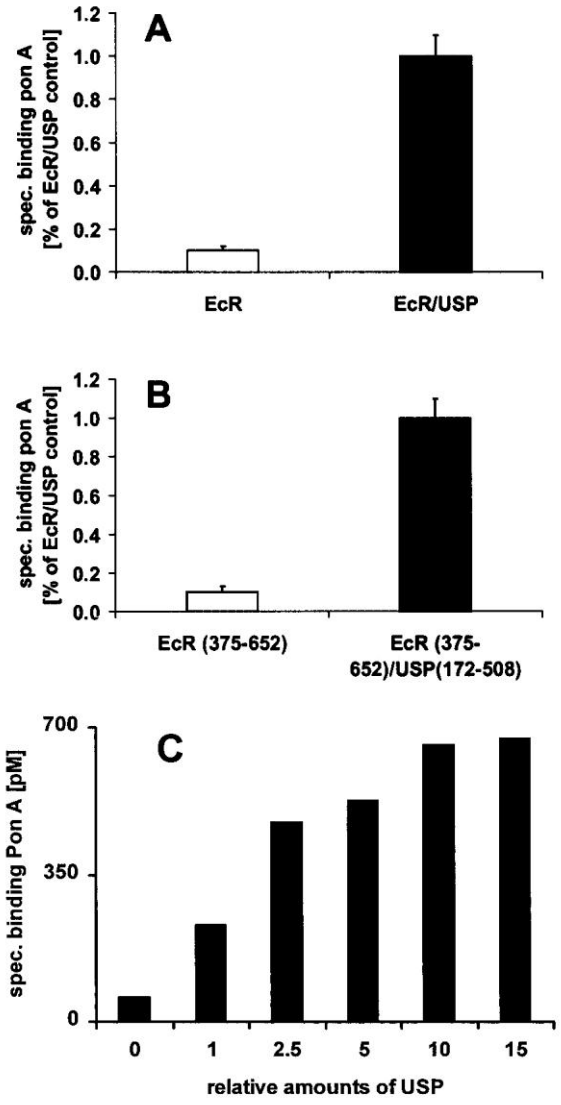

Fig. 4 Ligand Binding of Full-Length Receptors Obtained by *in vitro* Translation (A) and Fusion Proteins Gal4(AD)-EcR(375–652) and Gal4(DBD)-USP(172–508) expressed in *Saccharomyces* (B).
Given are mean values + SD, n=4. (C) Influence of USP concentration on ligand binding to EcR (SD < 20).

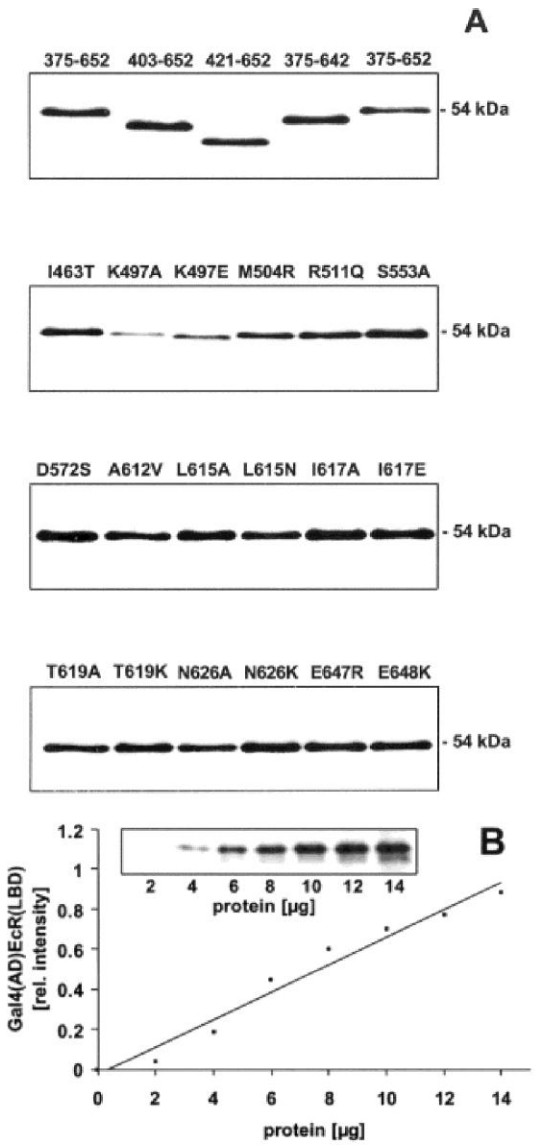

Fig. 5 Quantitative Determination of Receptor Concentrations.
(A) Western blots of GAL4 (AD) fusion proteins with EcR(375–652) wild-type and mutated receptor versions detected with a monoclonal antibody against GAL4 (AD). The expected shift in molecular mass is seen in the truncated versions.
(B) The calibration curve with wild-type EcR obtained by quantification of Western signals was used to determine the relative receptor concentrations in homogenates with mutated receptors. (r=0.98; SD<20%).

In the following ligand binding experiments and EM-SAs, the same ratio of receptor concentrations that was necessary to obtain maximal ligand binding of the wild-type heterodimer (10-fold increase compared to hormone binding to EcR) was used for all mutants. This standardization excludes the possibility of low ligand binding or dimerization, due to suboptimal concentrations of EcR and USP, being misinterpreted as a reduced ability for heterodimerization or hormone binding for EcR/USP. Since the receptor concentrations were the same in all tests, the differences between the mutations and the wild type reflect changes in hormone affinity.

Scatchard plots were not suitable because the con-centration of the both receptors had to be determined and no ligand – a prerequisite for determination of receptor concentrations – is known for the orphan receptor so far.

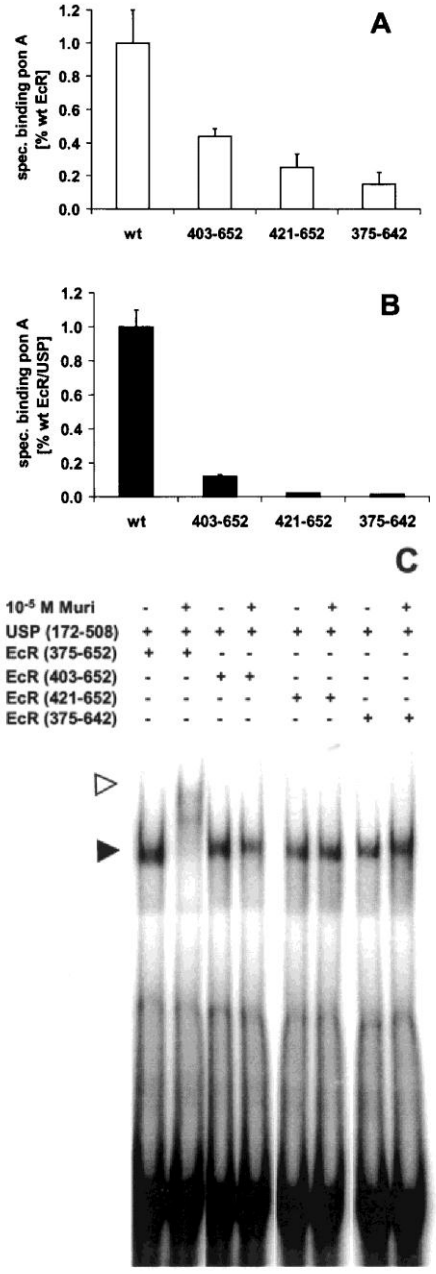

Fig. 6 [³H]-Ponasterone A Binding of GAL4 Fusion Proteins with (A) Truncated EcRs and (B) Corresponding Heterodimers with Wild-Type Gal4(DBD)-USP(172–508).
Specific [³H]-ponasterone A binding was expressed as percentage of hormone binding to wild-type EcR or EcR/USP fusion proteins (mean + SD, n= 4). (C) Gel mobility shift assays with GAL4-specific UAS and wild-type Gal4(DBD)-USP(172–508) and GAL4 (AD) fusion proteins (wild-type and truncations). In contrast to wild-type EcR(375–652) no ligand-dependent shift indicating heterodimerization is seen with any truncated receptor fragments in the presence of hormone. (▶) DNA/Gal4(DBD)-USP(172–508) complex. (▷) Hormone-dependent shift in the presence of GAL4 (AD) fusion proteins.

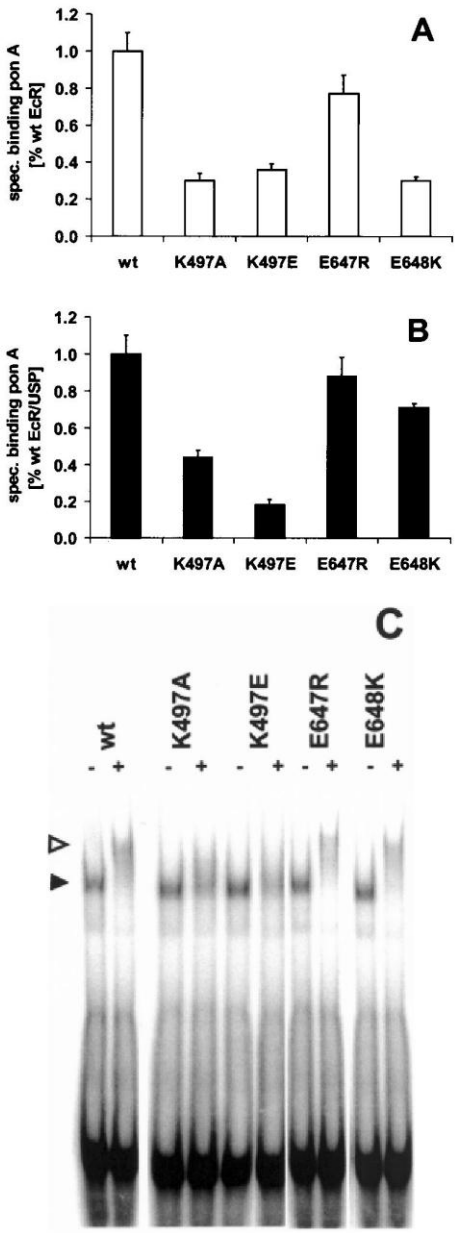

Fig. 7 Point Mutations of Amino Acids Involved in the Salt Bridge H4-H12.
(A) [³H]-Ponasterone A binding to Gal4(AD)-EcR(375–652) mutations and (B) corresponding heterodimers with wild-type Gal4(DBD)-USP(172–508). Specific [³H]-ponasterone A binding was expressed as percentage of hormone binding to the same concentration of wild type EcR or EcR/USP fusion proteins (mean + SD, n = 4). (C) Gel mobility shift assays with GAL4-specific UAS and wild-type Gal4(DBD)-USP(172–508) and GAL4 (AD) fusion proteins (wild type and point mutations). (+/-) Indicates the presence/absence of 10^{-5} M muristerone A. (▶) DNA/Gal4(DBD)-USP(172–508) complex. (▷) Hormone-dependent shift in the presence of GAL4 (AD) fusion proteins.

The C-Terminal Part of the Hinge Region and Helix 12 Is Required for Ligand Binding and Dimerization of the LBD

Examination of deletion mutants [EcR(403–652), EcR (421 –652)] lacking parts of the D-domain demonstrate that not only the E-domain but also the C-terminal part of the hinge region is essential for hormone binding (Figure 6A, B) and ligand-dependent heterodimerization (Figure 6C). EcR (375 - – 642) shows that helix 12 is indispensable for both functions of the receptor. This means that the EcR(375–652) fragment chosen is the

minimal sequence required for ligand binding and hormone-dependent heterodimerization. In all truncated versions, ligand binding is more seriously impaired in the case of EcR/USP as compared to EcR. This indicates that the in-creased hormone binding ability of the wild-type heterodimer is mediated at least partially by regions outside the canonical dimerization interface in helix 10. With EMSA, no ligand dependent formation of heterodimers is seen.

Destruction of the Salt Bridge between H4 and H12 Impairs Ligand Binding to EcR, But Hormone Binding Is Partially Rescued by Heterodimerization with USP

Not all amino acids of helix 12 are essential for receptor functions. Mutation of Glu at position 647 has only a minor effect on ligand binding to EcR; hormone binding of the heterodimer is in the normal range (Figure 7A, B). EMSA confirmed that ligand-dependent heterodimerization is not impaired (Figure 7C). In contrast, Glu648 is important for hormone recognition by the EcR, but ligand binding of the heterodimer is reduced to a lesser degree (Figure 7A, B), indicating that heterodimerization compensates to a certain extent the deleterious effect of this mutation. This was confirmed by EMSA, which shows that heterodimerization in the presence of ligand is in the normal range (Figure 7C).

In vertebrate nuclear receptors, there is a salt bridge from the conserved glutaminic acid in helix 12 to lysine in helix 4 (Wurtz et al., 1996). Mutation of Lys497 to alanine reduces ligand binding to EcR to a higher degree compared with EcR/USP. The electric charge is important for ligand binding of the heterodimer, since exchange of the basic amino acid by an acidic one in EcR^{K497E} diminishes ligand binding considerably (Figure 7A, B). According to EMSA, this is accompanied by insufficient dimerization. This indicates that Lys497 is involved in ligand binding and dimerization and may serve as a link to couple both functions. Disruption of the salt bridge alone is not responsible for this effect, since mutation of the second partner of the salt bridge E648 has only a moderate effect, although the charge of the amino acid was reversed. However, ligand binding to EcR is seriously impaired by mutation of both partners of the salt bridge. This may be a hint that not only the amino acids per se but also the integrity of the salt bridge is more important for ligand binding to EcR than for EcR/USP.

Functional Role of Amino Acids Lining the Ligand-Binding Pocket

Several amino acids are supposed to be involved in ligand binding according to the homology model presented by Wurtz et al. (2000). EcR^{I463T} does not impair ponasterone A binding to EcR and EcR/USP (Figure 8A, B), whereas EcR^{E476A} and EcR^{D572S} reduce ecdysteroid binding to EcR and the heterodimer to a considerable degree. Dimerization determined with EMSA is not affected (Figure 8C). M504 in helix 5 forms a hydrogen bond with the ligand according to the RAR based model (Wurtz et al., 2000). This amino acid is essential for hormone binding both to EcR and EcR/USP (Figure 8A, B), and is also important for ligand-dependent dimerization, which is abolished after mutation to arginine. EcR^{R511}, which forms a hydrogen bond with hormone according to the VDR-model (Wurtz et al., 2000), nearly lost its capacity for ligand binding to EcR after mutation to glutamine (Figure 8A). Ligand-dependent heterodimerization (Figure 8C) and ponasterone A binding of EcR^{R511Q}/USP is normal (Figure 8B) and indicates that the amino acid`s participation in ligand binding is different in EcR and EcR/USP. Hormone binding is rescued in the mutated receptor by interaction with the dimerization partner, as already shown for amino acids involved in the salt bridge H4-H12.

Mutations Presumably Affecting the 3-D Architecture of the Ligand-Binding Pocket

According to the homology model of Wurtz et al. (2000), amino acids at position 476, 553 and 572 have no direct contact to the ligand. Nevertheless, mutation of all three amino acids have an impact on ligand binding presumably by altering the 3-D architecture of the ligand-binding pocket (Figure 8). EcR^{S553} is another example of a suboptimal amino acid present in the wild type. The increase in ligand binding, however, is only observed with EcR, which demonstrates also that the conformation of the ligand-binding pocket is different in EcR and EcR/USP.

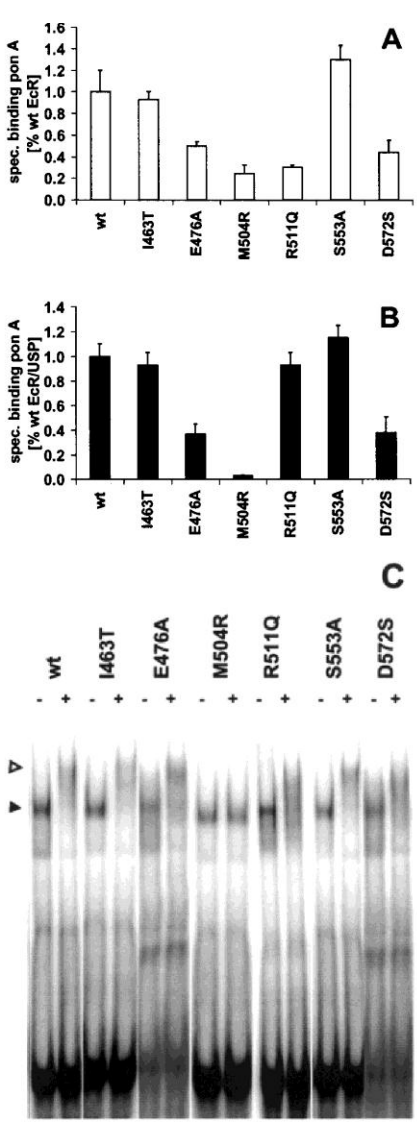

Fig. 8 Point Mutations of Amino Acids Lining the Ligand-Binding Pocket.
(A) [^{3}H]-Ponasterone A binding to Gal4(AD)-EcR(375–652) mutations and (B) corresponding heterodimers with wild type Gal4(DBD)-USP(172–508). Specific [^{3}H]-ponasterone A binding was expressed as percentage of hormone binding to the same concentrations of wild-type EcR or EcR/USP fusion proteins (mean + SD, n = 4). (C) Gel mobility shift assays with GAL4-specific UAS and wild-type Gal4(DBD)-USP(172–508) and GAL4 (AD) fusion proteins (wild type and point mutations). (+/-) Indicates the presence/absence of 10^{-5} M muristerone A. (▶) DNA/Gal4(DBD)-USP(172–508) complex. (▷) Hormone-dependent shift in the presence of GAL4 (AD) fusion proteins.

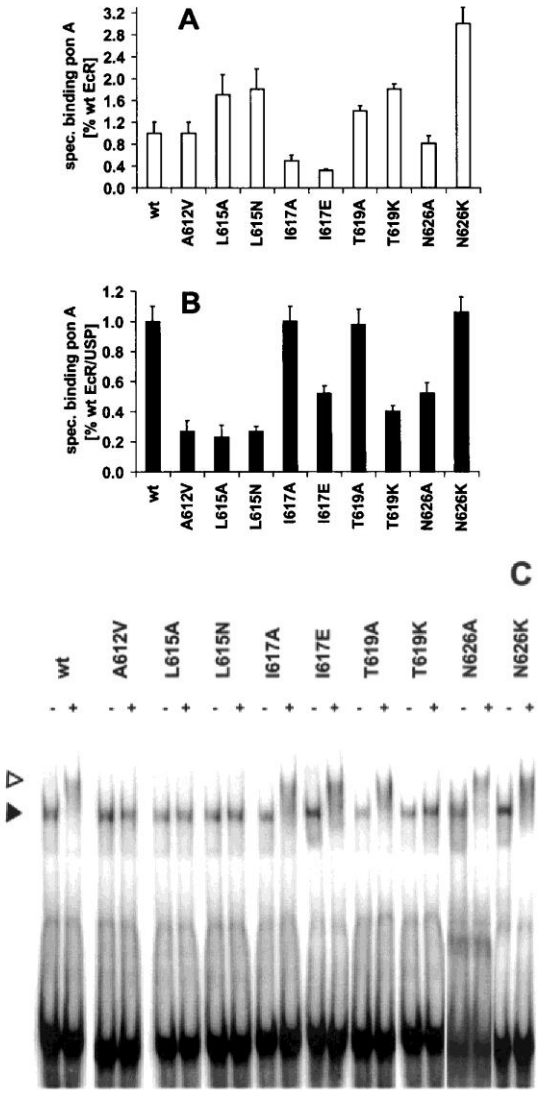

Fig. 9 Point Mutations of Amino Acids in Helix 10 or Loop H10-H11.
(A) [^{3}H]-Ponasterone A binding to Gal4(AD)-EcR(375–652) mutations and (B) corresponding heterodimers with wild-type Gal4(DBD)-USP(172–508). Specific [^{3}H]-ponasterone A binding was expressed as percentage of hormone binding to the same concentrations of wild-type EcR or EcR/USP fusion proteins (mean + SD, n = 4). (C) Gel mobility shift assays with GAL4-specific UAS and wild-type Gal4(DBD)-USP(172–508) and GAL4 (AD) fusion proteins (wild type and point mutations). (+/–) Indicates the presence/absence of 10^{-5} M muristerone A. (▶) DNA/Gal4(DBD)-USP(172–508) complex. (▷) Hormone-dependent shift in the presence of GAL4 (AD) fusion proteins.

Amino Acids in the Dimerization Interface (Helix 10) are Important for Ligand Binding to EcR/USP

EcR^{A612V}, EcR^{L615A}, EcR^{L615N} and EcR^{T619K} do not show ligand-dependent heterodimerization in EMSA, in accordance with the essential role of helix 10 for dimerization (Figure 9C). In all mutations this is accompanied by reduced ligand binding to EcR/USP (Figure 9A, B). Elongation of the aliphatic side chain leads to steric hindrance in EcR^{A612V}, whereas a shortened aliphatic side chain in EcR^{L615A} is not sufficient to allow efficient ligand-dependent heterodimerization. The length of the side chain seems to be more important than the hydrophilic proper-ties, since the introduction of asparagine at position 615 does not further impair the ligand binding in the presence of USP.

The Same Mutation Can Affect Ligand Binding to EcR and EcR/USP Differently

In addition to EcR^{R511Q} (Figure 8), several other mutations affect ligand binding to EcR and the heterodimer in opposite ways. Mutations of amino acids in the dimerization interface (helix 10) have a profound effect on

ligand binding. The ability to bind hormone is seriously impaired in heterodimers with EcR^{A612V}, EcR^{L615A}, and EcR^{L615N} (Figure 9) and confirms the important role of these amino acids in dimerization. Ligand binding to these EcR mutants in the absence of USP is either not affected or increased.

In contrast, I617 is involved in ligand binding to EcR, but the reduced capability for hormone binding in EcRI617A is compensated by heterodimerization. In EcR^{I617A}, a hydrophobic interaction is interrupted due to the shortened side chain of alanine. However, exchange of isoleucine by a charged amino acid in EcR^{I617K} impairs ligand binding also to the heterodimer. In contrast, introduction of a hydrophilic charged residue at position 619 even enhances ligand binding. This is most prominent for EcR, but is also seen with EcR/USP to a lesser degree.

N626 is situated in the loop between helix 10 and helix 11. Mutation to either lysine or alanine revealed that this amino acid is mainly engaged in ligand binding, since heterodimerization is still possible according to results obtained by EMSA, and ligand binding is only considerably impaired to the heterodimer with EcR^{N626A}. Hormone binding to EcR^{N626A} is only slightly reduced, but is considerably enhanced if the side chain is elongated by exchange of arginine with lysine, indicating that a suboptimal amino acid for hormone binding is present in the wild type. This is only apparent in ligand binding to EcR and not to the heterodimer. Since USP concentrations are critical for heterodimerization and hormone binding (Figure 4C), tests were repeated with higher USP concentrations to rule out the possibility that ligand binding to the heterodimer was underestimated due to insufficient USP concentrations. No increase was found with EcR^{N626K} (data not shown). These examples indicate that some amino acids are not optimal for ligand binding to the wild type but enable participation in a second receptor function (dimerization) at the expense of optimal ligand binding. They also clearly show that participation of individual amino acids differs in EcR and EcR/USP, thus demonstrating the allosteric effect of the heterodimerization partner.

Discussion

The general architecture of the ligand-binding domain, determined by X-ray analysis in nuclear receptors from vertebrates, revealed 11–12 helices and a ß-sheet arranged as an antiparallel sandwich (Wurtz et al., 1996). Prediction of the secondary structure using two different methods (Figure 2) showed that, despite of the low sequence identity (about 25% to RAR and VDR), basically the same structure was calculated for EcR. This was con-firmed by computer modeling of the ligand-binding pock-et using RAR and VDR as templates (Wurtz et al., 2000). This model allows prediction of ligand-binding properties in the middle of the ligand-binding domain, but the accuracy at the C- and N-termini is rather limited due to the low sequence similarity at both ends.

Fusion proteins with GAL4 were used in our investigation for several reasons. Due to the presence of GAL4-DBD, the influence of ligand on dimerization mediated only by ligand-binding domains of EcR and USP is measured by EMSA. With the same fusions two hybrid experiments were performed using the same mutations to study the influence of hormone on reporter gene activity (Lezzi et al., 2002; Bergman et al., unpublished). These tests allow comparing the effect of receptor mutations on dimerization quantitatively.

A tag is also essential for quantification of receptor concentrations by Western blotting, since no antibodies specific for the ligand-binding domain of EcR and USP are available. Quantification of receptor proteins is necessary; in order to rule out differences in the concentration of mutated proteins, which would lead to variations in the ligand binding tests. Scatchard analysis, which is traditionally used to eliminate the influence of variation in receptor concentration, cannot be used in the case of EcR/USP, because this method allows only determining the concentration of one receptor.

For reliable determination of ecdysteroid binding to EcR/USP the concentration and the ratio of both receptors is important and has to be monitored carefully. Therefore quantification of signals on Western blots was chosen, which is also more accurate than Scatchard analysis. Only in this case a single hormone concentration is sufficient to determine differences in receptor affinity.

The question remains whether the tag used for fusion with the receptor domains influences receptor proper-ties. The K_d values for full-length receptors EcR/USP and the corresponding fusion proteins are in the same range (M. Grebe, unpublished), therefore a major influence of the GAL4 moiety seems unlikely. This was also confirmed by comparison of purified Chironomus receptors with and without a GST-tag (Grebe and Spindler-Barth, 2002) and with Drososphila receptors fused to GFP (M. Grebe, unpublished). In all cases the K_d values are in the same range independent of the presence or absence of the tag. Meanwhile in vivo experiments confirmed the functionality of the GAL4 fusions with EcR- and USP-LBDs (Kozlova and Thummel, 2002). We conclude that hormone binding to EcR is an autonomous function of the ligand-binding domain and is not influenced by intramolecular interactions.

Drosophila ecdysteroid receptor dimerizes with USP in the absence of ligand (Yao et al., 1993) but binds also hormone in the absence of the heterodimerization partner (Bergman et al., unpublished; Lezzi et al., 2002).

Interaction with either ligand or USP enhances binding of the third partner considerably. Ligand binding to EcR indicates that the ecdysteroid receptor alone, and not only EcR/USP, may be functional and offers the possibility of an additional regulatory pathway for molting hormones in Drosophila. The ability of EcR to bind ligands in the absence of USP is species specific, since hormone binding to purified ecdysteroid receptor from Chironomus necessiates the presence of USP (Grebe and Spindler-Barth, 2002). This means that CtEcR can act only as ligand-independent transcription factor in this species. We cannot discriminate between weak hormone binding to DmEcR due to diminished formation of homodimers, or due to an altered ligand-binding pocket of EcR that is allosterically modified by heterodimerization to enhance ligand binding. However, the fact that USP can restore or diminish hormone binding with some EcR mutants to a certain extent compared to ligand binding to EcR alone indicates allosteric interactions between both dimerization partners. In any case, the diverse effects of the same mutations on ligand binding to EcR and EcR/USP shows that the configuration of the ligand-binding pocket is changed after heterodimerization. This is in accordance with vertebrate nuclear receptors; in RXR, the dimerization partner modifies the ligand binding of the corresponding partner to a high degree and thus allows or prohibits ligand binding (Glass, 1996).

In contrast to full-length receptors (Antoniewski et al., 1996; Vögtli et al., 1998; Elke et al., 1999), heterodimerization in the absence of a ligand cannot be demonstrated with EMSA using GAL4-fusion proteins, but selectively al-lows determination of hormone-dependent heterodimerization. Basal dimerization of EcR and USP in the absence of hormone can only be measured with the yeast two-hybrid system (Lezzi et al., 2002).

Truncations at either end of the ligand binding domain of EcR revealed that EcR (375–652) is the minimum sequence necessary for ligand binding. This means that the C-terminal half of the hinge region is also involved in ligand binding, although no direct contact to the ligand is possible. These results are in accordance with the data reported by Perera et al. (1999) obtained with Choristoneura EcR and are confirmed further by a mutation in the hinge region of EcR that was found in a hormone-resistant subclone of the Chironomus cell line (Zöllner, unpublished). In this case, full-length receptors were mutated to rule out reduction of ligand binding due to sterical hindrance, caused by the vicinity of the GAL4-moiety of the fusion protein. Prediction of the secondary structure (Spindler et al., 2001) revealed two additional helices in the C-terminal part of the hinge region, which may contribute to the stabilization of the 3 D-structure of the ligand-binding pocket. The hinge region and the N-terminal part of the E domain is the least conserved part of the ligand-binding region among insects. This leads us to assume that this region contributes to the species-specific differences in ligand binding and sensitivity to ecysteroids and diacylhdrazines (Dhadialla et al., 1998; Wurtz et al., 2000).

Truncation of the C-terminal helix 12 in EcR abolishes ligand binding completely. This is in contrast to several vertebrate receptors that still show ligand binding after removal of helix 12, with a couple of the exceptions: TR, whose affinity to the ligand is decreased after truncation (Wagner et al., 1995), and AR, which has lost binding capacity completely after mutation of an amino acid adjacent to H12 (Peters et al., 1999). This illustrates that, although the architecture of the ligand-binding domain is very similar in all nuclear receptors investigated so far, the function of individual receptor domains is different. Helix 12 mediates ligand-dependent

transactivation, induced by a change in the position of helix 12 after ligand binding and stabilization by a salt bridge between Glu648 in helix 12 and Lys497 in helix 4. Interruption of the salt bridge by mutation of either Lys497 or Glu648 nearly abolishes ligand binding to EcR. In opposition to the reduced ligand binding of EcR after mutation of Lys497 and Glu648, which is impaired to about the same degree, hormone binding of the heterodimer is only mildly affected in EcR^{E648K}. This shows that the lysine is essential for ligand binding to EcR and EcR/USP, but maintenance of the salt bridge is more important for ligand binding to EcR than to the heterodimer. Therefore, we assume that heterodimerization compensates partially for the loss of stabilization by the salt bridge. The allosteric effect of either USP or ligand to improve binding of the third partner can be explained by the stabilization of the same 3-D structure of the receptor complex by a salt bridge, or by heterodimerization.

Mutations of amino acids presumably lining the ligand-binding pocket lead to different changes in the functionality of this receptor domain. As shown in this report, EcR^{I463T} in Drosophila impairs ponasterone A binding only slightly, whereas in EcR of a hormone-resistant subclone of the Chironomus cell line isoleucine changed to leucine at the corresponding position is associated with a loss of hormone binding (Zöllner, unpublished; Grebe et al., 2000). Interestingly, the corresponding isoleucine is changed to threonine in wild-type EcR of Tenebrio (Mouillet et al., 1997). However, no ligand binding data are available from this species. Although ligand binding is more impaired in EcR^{E476A} and EcR^{D572S}, hormone-dependent dimerization is still possible. As expected, ligand-de-pendent dimerization is also abolished in a mutated receptor that cannot bind ecdysteroids any more like EcR^{M504R}. In contrast, defective ligand binding of EcR^{R511Q} can be rescued by dimerization. Arginine at position 511 is a very conserved amino acid, which mediates hormone binding by the formation of a hydrogen bond with the ligand also in vertebrate nuclear receptors like progesterone receptor (Letz et al., 1999). Mutation of this amino acid clearly demonstrates the interdependence of different receptor functions.

According to the homology model presented by Wurtz et al. (2000), the amino acids at positions 476, 553 and 572 are solvent exposed and cannot contact the ligand directly. Nevertheless, either reduced ligand binding is observed after mutation (EcR^{E476A}, EcR^{D572S}), or hormone binding to EcR is enhanced in the absence of USP (EcR^{S553A}). We speculate that these effects are indirect and are due to changes in the architecture of the ligand-binding pocket.

Helix 10 is important for dimerization (Bourget et al., 1995). This region is rich in leucine and other hydrophobic amino acids both in EcR and USP, although a typical leucine zipper motif or LXXLL sequence is missing. The mutants EcR^{A612V}, EcR^{L615A} and EcR^{L615N} confirm the importance of hydrophobic interactions for dimerization. The elongation of the hydrophobic amino acid residue in A612V impairs dimerization by steric hindrance. In contrast, replacement of alanine by glutamine in Bombyx mori EcR enhances dimerization (Suhr et al., 1998). The length of the hydrophobic site chain is also important for L615 in contrast to EcR^{I617A}. In this case, replacement by a charged amino acid is necessary to impair dimerization.

Besides these more or less specific effects on ligand binding and dimerization observed after mutation of a single amino acid, we have to be aware that changes in the general architecture of the ligand-binding pocket may also contribute to the differences in receptor function. This is most prominent for the D-domain, which has no direct contact to the ligand and the solvent exposed amino acids at positions 476, 553 and 572. It may also contribute to the effects described for other point mutations. These general effects will be described in detail by Bergman et al. (unpublished).

Generally, ponasterone A binding to the heterodimers goes in parallel with ligand dependent heterodimerization measured with EMSA, confirming that the same complex is measured with both methods. We never observed ligand binding significantly exceeding that of wild-type heterodimers. However, considerably enhanced galactosidase induction was found both in the absence and in the presence of hormone in the two-hybrid system (EcR^{K497A} and EcR^{I617E}, EcR^{S553E}, EcR^{D572S}, EcR^{T619A} and EcR^{T619K}, EcR^{N626A} and EcR^{N626K}) (Bergmann et al., unpublished) and indicates that additional factors in the yeast cell modify either the interaction between both

receptors and their trans-activation.

In contrast, enhanced ligand binding to mutated EcR compared to the wild type was found several times. In EcR^{L615A}, EcR^{L615N}, and EcR^{T619K} increased hormone binding to EcR is accompanied by impaired heterodimerization. Obviously reduced affinity to wild-type EcR is accepted in order to enable the mediation of both functions by the same amino acid. This demonstrates that enhanced flexibility of hormonal regulation by coupling of the two receptor functions is preferred over an optimal single function.

Different effects of the same mutation, e.g. EcR^{R511Q}, EcR^{N626K}, and EcR^{E648K}, on ligand binding to EcR and EcR/USP show that the contribution of the same amino acid in ligand binding to EcR in the presence and absence of USP is not identical. This means that the 3-D structure of the ligand-binding pocket is changed by heterodimerization. The diverse effects of amino acids outside the dimerization interface on hormone binding to EcR and the heterodimer with USP indicate conformational changes of major parts of the ligand-binding pocket induced by heterodimerization, and are in accordance with the changes in the 3-D structure reported for apoRXR and holoRAR (Bourget et al., 1995; Renaud et al., 1995).

Wurtz et al. (2000) described homology models using RAR and VDR as templates, which define amino acids lining the ligand-binding pocket. Our results basically confirm the proposed model for hormone binding, but demonstrate that several additional amino acids are en-gaged in hormone binding as well. This involves mainly amino acids at the dimerization interface in helix 10, and the salt bridge and the AF2 domain in helix 12. In addition, it was shown that some amino acids lining the binding-binding pocket or situated in helix 10, which is sup-posed to be involved in dimerization, are engaged in both receptor functions.

Ligand binding is a dynamic process. The high dissociation rate constants (Yund et al., 1978; Turberg et al., 1988) between hormone and ecdysteroid receptor show that the intra- and intermolecular allosteric interactions associated with ligand binding are easily reversible. Kosztin et al. (1999) calculated that the main pathways for ligand entry and ligand exit were different in RAR. Both paths do not involve helix 12. 20-OH-ecdysone, the most important naturally occurring ligand of the ecdysteroid receptor, has no charge like retinoic acid and is much more hydrophilic than vitamin D. These properties will certainly affect the mechanism of hormone binding. In contrast, ligand binding to EcR depends on the participation of helix 12 and the amino acids involved in the salt bridge. This points to a different mechanism for association and dissociation of ligands to the ecdysteroid receptor compared to the RAR-based model of Kosztin et al. (1999), and is currently under investigation.

Materials and Methods

Yeast Strain

Saccharomyces cerevisiae strain Y190 (Harper et al., 1993) was cultured according to the manufacturer (Clontech yeast protocols handbook PT3024-1; Clontech Laboratories, USA). Yeast transformation was performed with lithium acetate (Guthrie et al., 1991). Transformants were selected by auxotrophy for tryptophan (pAS2-1) and leucine (pACT2), respectively.

Construction of Yeast Expression Plasmids

DNA encoding the C-terminal part of the D-domain and the E-domain (LBD) of Drosophila ecdysteroid receptor was cloned into the expression vector pACT2 (Li et al., 1994), resulting in a Gal4(AD)-EcR(LBD) (AD: activating domain) fusion protein. For expression of Gal4(DBD)-USP(LBD) (DBD: DNA binding domain) the C-terminal part of the D-domain and the E-domain of Drosophila ultraspiracle (Figure 1) was cloned into the vector pAS2-1 (Harper et al., 1993). GAL4 expression plasmids and the corresponding plasmids with EcR and USP wild type and mutants created by site-directed mutagenesis were kindly provided by Dr. Lezzi (ETH Zürich, Switzerland).

Preparation of Yeast Extracts

Single colonies (not older than 4 days) of yeast transformants carrying the expression plasmids either for Gal4-

AD-EcR or GAL4-DBD-USP wild types or the corresponding mutants were picked and cultured at 30°C overnight in 5 ml selective medium containing 2% glucose with shaking (150–200 rpm). The overnight cultures were vigorously shaken to disperse the cells thoroughly, diluted in 50 ml YPD medium (20 g/l peptone, 10 g/l yeast extract, 2% glucose) and grown under the same conditions until the OD600 reached 0.6 to 0.8. If not stated otherwise, the following steps of preparation were done on ice. Yeast cells were harvested by centrifugation (1500 g, 5 min, 4°C) in prechilled tubes. Cell pellets were washed with 50 ml ice-cold wash buffer (20 mM HEPES, 20 mM NaCl, 20% glycerol, 1 mM EDTA, 1 mM 2-mercaptoethanol, pH 7.9) and transferred into plastic tubes, supplemented with a steel ball and frozen in liquid nitrogen for about 20 s. The frozen pellets were disrupted for 2 min at 2000 rpm using a Micro-dismembrator S (B. Braun Biotech International, Melsungen, Germany). After thawing homogenates were diluted with binding buffer [20 mM HEPES, 20 mM NaCl, 20% glycerol, 1 mM EDTA, 1 mM 2-mercap-toethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.9] supplemented with a mixture of protease inhibitors (aprotinin, leupeptin, pepstatin, benzamidine, antipain, chymostatin; final concentration 2 µg/ml each) immediately before use. After short treatment with ultrasonic power (Branson Sonifier, B-12, Bran-son, Danbury, USA) using a microtip (2 × 2 s, 90 W) the samples were centrifuged (100 000 g, 1 h, 4 °C). PMSF (final concentration 1 mM) was added to the supernatants. The extracts were frozen in aliquots at –80°C until use for ligand binding assays, Western blot analysis and electrophoretic mobility shift assays (EMSA).

Western Blot and Quantitative Determination of EcR and USP Fusion Proteins

Yeast cell extracts were diluted with sample buffer (final concentration 100 mM Tris, 3% SDS, 2% 2-mercaptoethanol, 10% glycerol, 0.05% bromophenol blue, pH 8.8) and boiled for 3 min (Laemmli, 1970). 10–20 µg protein per lane (Bradford, 1976) were loaded onto a SDS-gel (0.6× MDE gel solution, Boehringer, Ingelheim, Germany; AT Biochem., Minigel Twin, 8.6×7.2× 0.1 cm, Biometra, Göttingen, Germany). Western blotting was performed according to Khyse-Andersen (1984). Gels were electroblotted on nitrocellulose membranes (BA 85, 45 µm pore size, Schleicher and Schuell, Dassel, Germany). The membranes were soaked in blocking buffer (5% milk powder, 1 % fat in 20 mM Tris-HCl, 137 mM NaCl, 0.1 % Tween 20, pH 7.6, 0.02% Thimerosal) and probed either with Gal4-AD monoclonal mouse antibody (# 5398 – 1, Clontech Laboratories) or Gal4-DBD polyclonal rabbit antibody (# sc-577, Santa Cruz Biotechnology Inc, Santa Cruz, USA) diluted in blocking buffer 1:5000 (Gal4-AD) and 1:100 (Gal4-DBD), respectively. To detect specific Western signals per-oxidase conjugated secondary antibodies diluted in TBS (0.1 % Tween 20) were used (1:1000 anti-mouse IgG, or 1:500 anti-rabbit IgG, both from Sigma). After visualization with an ECL detection kit (Amersham) according to the instructions of the supplier specific signals were scanned (Scanner JX-325, Sharp, 600 dpi, software ViceVersa Scan 1.2, Krystec EDV, Norderstedt, Germany) and analyzed with an image analysis system (PHORETIX, Nonlinear Dynamics, LTD, Newcaste, UK; resolution 600 dpi, corresponding to 42 µm2). The intensity of a given band was quantified and taken as measure for EcR or USP concentration. The linearity was previously verified by calibration (Rauch et al., 1998).

Ligand Binding Assays

Yeast cell extracts were diluted to an appropriate protein con-centration (Bradford, 1976) with binding buffer and supplemented with protease inhibitors (see above) immediately before use. Ligand binding was determined with [^{3}H]-ponasterone A (specific activity 7.9 TBq/mmol; kind gift of Dr. H. Kayser, Syngenta, Switzerland) using a filter assay as already described in detail (Turberg and Spindler, 1992) The appropriate amounts of yeast extracts with EcR and USP fusion proteins (determined by quantification of Western blot signals) were mixed together and incubated with 4 – 5 nM [^{3}H]-ponasterone A for 1 h at room temperature. For each sample the nonspecific binding determined by addition of 0.1 mM non-labeled 20-OH-ecdysone was subtracted. The purity of [^{3}H]-ponasterone A was checked routinely by HPLC analysis before use. Routinely two different extracts were tested in duplicate. Ligand binding data of mutated receptors were expressed as % of wild type hormone binding (= 100%).

Electrophoretic Mobility Shift Assay (EMSA)

The oligonucleotides dgal 1 (5’-GATCGCACAGTGCCGGAG-GACAGTCCTCCGGTTCGAT-3’) and dgal (5’-GATCATCGAAC-CGGAGGACTGTCCTCCGGCACTGTGC3’) were formed by annealing 5’-extensions

with the sequence GATC labeled with [α-^{32}P]-dCTP by fill-in reaction with Klenow polymerase. The labeled oligonucleotides were used as probe for binding to the fusion proteins.

The reaction mix contained binding buffer [20 mM HEPES, pH 7.4, 100 mM KCl, 5% (v/v) glycerol, 2 mM dithiothreitol, 0.1 % NP-40], yeast cell extracts with the EcR or USP fusion proteins, 1 µg non-specific competitor poly(dIdC) and approximately 10 fmol labeled oligonucleotide. 10^{-5} M muristerone A (final concentration) was used, where indicated. The reaction mix was incubated at room temperature for 30 min and separated at 10 V/cm on a 5% nondenaturing polyacrylamide gel in 0.5× TBE (45 mM Tris, 45 mM boric acid, 0.5 mM EDTA, pH 8.0) for 2 h. Gels were dried, scanned with a Phosphorimager and evaluated with ImageQuant™ software package (Molecular Dynamics, Sunnyvale, USA).

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